

GENETICS OF PEROXIDASE ISOENZYME POLYMORPHISMS  
IN MAIZE (ZEA MAYS L.)

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## INTRODUCTION

The heterogeneity of enzymes which catalyze the same reaction has been examined extensively, and several reviews have been written on the subject (Kaplan 1963; Wilkinson 1966; Latner 1967; and Shannon 1968). Within any organism, organ, or tissue, most enzymes exist in multiple forms which are detectable by chromatography, electrophoresis, gel filtration, catalytic properties, sedimentation, immunochemistry, or combinations of these methods. Gel electrophoresis as first described by Smithies (1955) provides a simple method of observing molecular heterogeneity from relatively crude extracts. With this technique, proteins are separated on the basis of their net charge and size; a single amino acid substitution can result in an altered electrophoretic mobility (Ingram 1957). The enzymes are visualized on the gel as zones of activity by means of histochemical staining procedures and the resulting pattern of bands has been designated a "zymogram" (Hunter and Markert 1957).

Markert and Møller (1959) proposed that these multiple forms of a protein within a species, exhibiting the same enzymatic specificity, should be called "isozymes". At the same time they recognized the difficulty in applying this term to those enzymes with broad specificities, such as esterase, peroxidase, and phosphatase. The Standing Committee on Enzymes of the International Union of Biochemistry later proposed that the term "isoenzyme" be used in preference to "isozyme" (Wilkinson 1966). No distinction has been drawn here between these two terms, and they are considered to be completely synonymous. The term "peroxidase isoenzyme" (or "isozyme") as used in this dissertation refers

to those zones on a starch gel which show peroxidase activity under the specific conditions described.

Beadle and Tatum in 1941 proposed their classic one-gene one-enzyme theory, which states that a single gene determines phenotype through its effect on the production of a single enzyme. Using a series of Neurospora mutants, they proved that many biochemical reactions were under the control of single alleles. In higher plants, most phenotypes represent the end result of many biochemical steps which must be elucidated before biochemical studies of the Neurospora type can be performed. The isoenzyme technique is a quick and easy means of visualizing some enzymes as a stained zone or zones on paper, starch, polyacrylamide, or agar. It has been established that proteins are coded for by genes, and hence this biochemical phenotype, the isoenzyme, may represent the direct product of a gene. Enzymatic mutants thus provide a system in higher organisms whereby the genetics of enzyme production can be readily determined. Since forms of an enzyme differing in as little as one amino acid residue can be detected by electrophoresis, genetic analysis of isoenzymes will no doubt prove to be important in further studies of gene action.

Genetically, maize is the most extensively studied higher plant, in which several hundred mutants have been described (Neuffer, Jones and Zuber 1968). Eighteen isoenzyme loci have been documented in maize, including genes controlling polymorphisms for esterases, leucine aminopeptidases, catalases, alcohol dehydrogenases, catechol oxidases, phosphatases and aminotransferases. The inheritance of these isoenzymes ranges from simple monogenic systems to complex interacting gene systems,



and these will be discussed in the literature review section. Isoenzyme genetics has not yet been studied in any other higher plant with the exception of peas, where there has been a suggestion that the production of each of two esterase bands is determined monogenically (Frankel and Garber 1965).

Peroxidase is a heterogeneous enzyme with a rather broad specificity. The chemical structure and catalytic properties are known in great detail, but the *in vivo* functions remain to be elucidated. Several roles have been suggested for peroxidases. They appear to be involved in the lignification process; the intensity of lignification and peroxidase activity are correlated. Peroxidases appear also to play a role in auxin synthesis and breakdown, and are considered to be essential components of the indoleacetic acid oxidase system in plants, suggesting that enzymes having peroxidase activity are intimately involved with the growth process. Another postulated role for peroxidase has been in the disease resistance mechanism, since an infection by most pathogens results in a rapid increase in peroxidase and other oxidative enzymes. Clearly no single role can be assigned to plant peroxidases, and it might be expected that multiple forms contribute to this polymorphic status.

Genetic data for peroxidase isoenzyme loci in maize or other species had not been published prior to the commencement of this work. The purpose of this investigation was to examine the extent of peroxidase polymorphisms in strains and tissues of Zea mays and to determine the basis of inheritance of observed genetic polymorphisms.

## LITERATURE REVIEW

### Isoenzyme Loci in Maize

Genetic studies with the esterases of corn were begun by Schwartz (1960a). The first esterase studied was a cathodally moving isoenzyme which he called the pH 7.5 esterase, or the  $E_1$  esterase (1960b). This locus was found in both endosperm and seedling tissues. Seven alleles were subsequently described (Schwartz, Fuchsman and McGrath 1965), and the enzyme determined to be active as a dimer, with hybrid enzymes formed in all heterozygotes. The timing of action of the  $E_1$  esterase alleles was found to be controlled by some element located close to the  $E_1$  structural gene (Schwartz 1962). The regulatory gene was believed to interact with certain of the  $E_1$  alleles, causing precocious cessation of enzyme production in the endosperm. Glyceraldehyde treatment eliminated charge differences among the  $E_1$  allelic isoenzymes, but the mechanism for this was not determined (Schwartz 1965). Schwartz concluded later (1967) that the allelic isoenzymes differed in conformation, and that the difference in electrophoretic behaviour was due to differential masking of charged groups.

Schwartz (1964) reported on a second esterase system in maize which he called  $E_3$ . The  $E_3$  bands moved cathodally and there were two variants, a fast and a slow, with a hybrid band being formed in heterozygotes. The  $E_3$  locus consisted of two alleles, and the locus was not linked to  $E_1$ .

Harris (1966) described the  $E_4$  esterases, anodal isoenzymes in the seedling root. He implied that this was a five allele system at a single locus on Chromosome III (Harris 1968). Macdonald (1969) also

studied the  $E_4$  esterases and determined that the structural  $E_4$  locus was controlled by an independently segregating locus. This system is thus analogous to the  $E_1$  esterases, where certain alleles of a structural locus interact with a regulatory locus.

Macdonald (1969) described seven other esterase loci in maize. The  $E_5$  esterases were found to be under the control of two independently segregating loci  $E_5$ -I and  $E_5$ -II, acting with recessive complementation. Esterases  $E_6$ ,  $E_7$ ,  $E_8$ ,  $E_9$ , and  $E_{10}$  were each governed by a monogenic system with two or three alleles. Most of Macdonald's genetic analyses were carried out on seedling root tissue or pollen, although the  $E_{10}$  locus was defined for studies of endosperm.

Two leucine aminopeptidase loci were reported active in maize endosperm (Beckman, Scandalios, and Brewbaker 1964a). These were called LpA and LpD. Each locus consisted of two alleles, a fast and a slow, acting with no dominance. The two genes were found to be linked.

Beckman, Scandalios, and Brewbaker (1964b) also reported a catalase locus (Cat) in maize endosperm consisting of two alleles responsible for a fast and a slow band. Three hybrid enzyme bands were present in typical heterozygotes, suggesting that catalase was active as a tetramer.

Two alcohol dehydrogenase loci,  $Adh_1$  and  $Adh_2$  were discovered in maize endosperm and scutellum by Schwartz (1966). Each locus had two alleles, and the enzyme was active as a dimer. These two genes were found to be duplicate genes; the  $Adh_1$  locus being much more active than the  $Adh_2$ . Scandalios (1967) also studied two alcohol dehydrogenases which he called Adh-1 and Adh-2. He concluded that Adh-1 existed as a dimer whereas Adh-2 existed as a monomer.

One aminotransferase or transaminase isoenzyme locus ( $Ta_1$ ) with two alleles was described in corn seedling tissue by Macdonald (1969). This enzyme appeared to be active as a dimer, with hybrid bands appearing in heterozygotes.

A catechol oxidase (Cx) has been described in seedling shoot tissue (Pryor 1969). This locus which was located on Chromosome X, had three alleles and appeared to be under the influence of a modifier.

Brown and Allard (1969) presented data on an alkaline phosphatase found in seedling tissues. They had evidence for three co-dominant alleles at a single locus which they designated  $Phos_4$ .

Hamill (1968) described a peroxidase locus with two alleles in corn seedling root, and further evidence (Brown and Allard 1969) suggested that there were three alleles.

In summary, the genetics of isoenzymes of eight different enzyme systems has been analyzed in maize, with a total of eighteen loci described.

### Peroxidase: The Enzyme

Definition and Occurrence: Peroxidase comprises a widespread and versatile class of enzymes capable of catalyzing many varied reactions. There is clearly no single entity as "plant peroxidase", but instead many different peroxidases, each one carrying out a specific reaction via a somewhat similar peroxidatic mechanism.

Peroxidase belongs to the class of enzymes known as oxidoreductases, which includes all enzymes capable of catalyzing oxidation--reduction reactions (Dixon and Webb 1965), and has been given the systematic name of Donor:  $H_2O_2$  oxidoreductase, and EC number of 1.11.1.7. By definition

a peroxidase is an enzyme catalyzing the oxidation of compounds such as phenols, aromatic amines, leuco-dyes, ascorbic acid, indole, or organic ions, utilizing hydrogen peroxide as the oxidant. Both the peroxide and the compound undergoing oxidation have been referred to in the literature as the substrate. To avoid confusion, the hydrogen peroxide should be referred to as the oxidant and the compound undergoing oxidation as the hydrogen donor (Saunders, Holmes-Siedle and Stark 1964). Many molecules, including those listed above, are capable of serving as the hydrogen donor. Some peroxidases have been given specific names according to the specific compound oxidized in the reaction, for example, chloroperoxidase which oxidizes the chloride ion. In other cases, peroxidases have been named according to the tissue from which they have been isolated, for example, myeloperoxidase from white blood cells.

Peroxidase must be distinguished from both oxidases and catalases. An oxidase catalyzes a reaction utilizing oxygen as the oxidant, rather than hydrogen peroxide. Catalase is a specific enzyme whose function it is to destroy hydrogen peroxide. In this reaction, hydrogen peroxide serves both as the oxidant and hydrogen donor.

The first report of a peroxidase reaction was made by Schonbeim in 1855 (Saunders, Holmes-Siedle and Stark 1964) when he noted that the oxidation of certain organic compounds by hydrogen peroxide could be catalyzed by substances present in plants and animals. The name peroxidase was given to this substance by Linoissier in 1898 who isolated peroxidase from pus. Following this, peroxidase has been found to occur universally throughout plant and animal kingdoms.

Peroxidase has been reported in viruses, bacteria, fungi, and through higher plants. In animals it has been reported at all evolutionary levels from protozoa to man. An extensive listing of the sources of peroxidase is given in Chapter 2 of Saunders, Holmes-Siedle and Stark (1964). In plants, peroxidase activity is found in all tissues, although in varying amounts. Commonly, activity is highest in roots and lowest in dry seed (Goren and Goldschmidt 1966). Horseradish root is particularly rich in peroxidase and is used as the source for commercial preparations. Peroxidases all catalyze the same reaction but differ greatly in their optimum requirements. In this literature review emphasis will be placed on plant peroxidases since all results in this study were derived from one plant species, Zea mays L.

Structure and Mechanism of Action: Peroxidase has been obtained in a crystalline state from several plant sources. Some of these are: horseradish (Theorell 1942a), Japanese radish (Morita and Kameda 1961), wheat (Tagawa, Shin and Okunuki 1959), turnip (Hosoya 1960a, 1960b, 1960c), and pineapple (Beaudreau and Yasunobu 1966). The chemical structure and kinetics of the peroxidase molecule have been the subject of study of many investigators (Theorell 1942a, 1942b, 1942c, 1943a; Theorell and Paul 1944; Cecil and Ogston 1951; Keilen and Hartree 1951; Shannon, Kay, and Lew 1966; Kay, Shannon, and Lew 1967; Strickland, Kay, Shannon, and Horwitz 1968). Peroxidase molecules have been found to comprise three components, a colorless protein (apoenzyme) consisting of at least sixteen amino acids, an iron porphyrin prosthetic group (protoporphyrin IX), and a carbohydrate fraction. The molecular weight of plant peroxidase is in the range 40,000 - 54,000, the 40,000 value being

that reported for horseradish peroxidase (Shannon, Kay and Lew 1966) and the 54,000 value that for Japanese radish (Saunders, Holmes-Siedle and Stark 1964). Peroxidase as isolated from horseradish had an iso-electric point of 7.2 and a solubility of 5 gm per 100 ml of water. It was stable over a wide range of pH values (3.5 - 12.0) and maintained activity over long periods of storage. The peroxidase molecule was found to be folded and held together by disulphide bonds. The means of attachment of the carbohydrate moiety has not been determined, nor has its role in the properties of the enzyme (Shannon, Kay, and Lew 1966).

Theorell (1942a) first suggested that peroxidase was composed of more than one component. Using paper chromatography and electrophoresis, Jermyn (1952) found four different components in commercial horseradish peroxidase, and Jermyn and Thomas (1954) observed five peroxidase components in horseradish root juice. Markert and Möller (1959) reported electrophoretic differences in commercial horseradish peroxidases: seven cathodal zones and three anodal. Klapper and Hackett (1965) isolated and characterized five major components which were found to be similar in size, absorption spectra, enzymatic activity, and amino acid composition. Shannon, Kay, and Lew (1966) isolated seven components from horseradish peroxidase. They found that these seven could be separated into two groups on the basis of their electrophoretic migration, chromatographic behaviour, spectrophotometric properties, and amino acid and carbohydrate composition. Isoenzymes within each of these two groups possessed similar catalytic properties, but varied in other properties such as pH optima, specific activities, apparent  $K_m$  values, and affinity toward inhibitors (Kay, Shannon, and Lew 1967).

Using electrophoretic techniques, the peroxidase of many other plant species in addition to horseradish has been found to exist in multiple forms. Some of these are: wheat (Tagawa, Shin, and Okunuki 1959), beans (Racusen and Foote 1966), Japanese radish (Shimizu and Morita 1966), Petunia (Hess 1967), barley (Giacomelli 1967), tobacco (Hart and Bhatia 1967), soybean (Buttery and Buzzell 1968), tomato (Evans and Aldridge 1965), peas (Macnicol 1966), pumpkin (Dvorak and Cernohirska 1967), and corn (McCune 1961). As well, pineapple, papaya, Leucaena spp., citrus, and many other plants studied in this laboratory (Unpubl.) have been observed to possess several peroxidase isoenzymes. The whole subject of plant isoenzymes has been reviewed by Shannon (1968).

By definition, a peroxidase catalyzes the oxidation of a compound, utilizing hydrogen peroxide as the oxidant. Peroxidase activity is present in nearly all metal or heme- containing proteins (Hosoya and Morrison 1967). In order for a peroxidase to be considered a typical or true peroxidase, it must be a heme protein with a high specific activity. All true peroxidases act via the same mechanism, with the oxidation--reduction reactions taking place at the iron site of the heme portion of the molecule.

The overall reaction very simply stated is as follows:



The reaction takes place in steps, with the formation of intermediate compounds, which in many cases are short-lived and hence have not been identified. The intermediate compounds are colored however, allowing the reactions to be followed spectrophotometrically. The



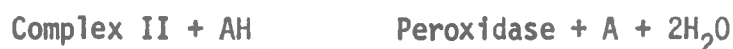
mechanism of oxidation by peroxidase is thought to proceed by the following steps. First the peroxidase reacts with hydrogen peroxide to form Complex I.



Complex I is a green compound with maximum absorption at 410 mμ, and in the presence of a hydrogen donor (AH) is converted to Complex II.



Complex II is a red compound, absorption maximum 418 mμ, which then reacts with the free radical, regenerating the peroxidase molecule, an oxidized donor, and water.



With an excess of hydrogen peroxide, red Complexes III and IV are formed. These are catalytically inactive and hence the reaction is inhibited when they are formed.

The initial union of peroxidase with hydrogen peroxide has historical significance in that it was the first observed example of the enzyme-substrate complex of Michaelis - Menton (Saunders, Holmes-Siedle, and Stark 1964).

Specific Peroxidases: Horseradish peroxidase has been discussed in preceding sections as an example of a typical plant peroxidase. Other peroxidases which should be reviewed are chloroperoxidase, myeloperoxidase, lactoperoxidase, iodide peroxidase, fatty acid peroxidase, and cytochrome c peroxidase.

Chloroperoxidase has been extracted from a mold Caldariomyces fumago. It was purified and crystallized and found to contain

ferriprotoporphylin IX, has a minimum molecular weight of 40,000, and an extinction coefficient of  $2.6 \times 10^5 \text{ cm}^{-1}$  at 408 m $\mu$  (Morris and Hager 1966). In structure and composition this enzyme was found to be very similar to both horseradish peroxidase and Japanese radish peroxidase. In the presence of a suitable acceptor molecule, chloroperoxidase utilized chloride, bromide, and iodide ions to catalyze the formation of a carbon--halogen bond. Fluorine was not a substrate, but acted instead as an inhibitor (Hager, Morris, Brown, and Eberwein 1966). Horseradish peroxidase and Japanese radish peroxidase both have the ability to oxidize the iodide ion but cannot carry out the chlorination or bromination of chloroperoxidase. This suggested that the active site of chloroperoxidase was different than that of horseradish or Japanese radish peroxidase.

Myeloperoxidase, also called verdoperoxidase, is present in limited amounts in white blood cells from various sources. Although it was observed to have many of the properties of true peroxidases, spectrophotometric studies indicated that the prosthetic group of myeloperoxidase was different from any known heme protein (Schultz and Shmukler 1964). It appeared that this peroxidase had two heme groups per molecule, and the two were either different or were surrounded by different microenvironments (Ehrenberg 1962). Myeloperoxidase has been separated into ten components by free flow electrophoresis, and Schultz, Felberg and John (1967) have postulated that the native enzyme might consist of two of these components held together by ionic linkage.

Lactoperoxidase was isolated from cow's milk by Theorell (1943b), who determined that it also was a heme protein and was different from

myeloperoxidase. Bovine lactoperoxidase was further purified and partially characterized by Rombauts, Schroeder, and Morrison (1967). They determined that its molecular weight was about 77,500, and suggested on the basis of peptide maps of the soluble tryptic peptides, that lactoperoxidase consisted of two nearly identical subunits. This indicated that lactoperoxidase was unique; other peroxidases are not generally considered to be subunit molecules.

Iodide peroxidase has been extensively studied in animal tissues, particularly in the thyroid DeRobertis and Grasso 1946; Alexander 1959; DeGroot and Davis 1961; Klebanoff, Yip, and Hessler 1962; Yip 1965; Yip and Hadley 1966; Hosoya and Morrison 1967). It can however, be readily demonstrated that plant peroxidases have the ability to oxidize the iodide ion. Iodide peroxidase catalyzes the iodination of tyrosine to mono- or diiodotyrosine (Alexander 1959) but a hydrogen peroxide generating step is necessary first. Agents which stimulate this reaction do so through the formation of hydrogen peroxide (DeGroot and Davis 1961). In the presence of the proper substances, myeloperoxidase and lactoperoxidase also catalyze the iodination of tyrosine.

It has been determined that long chain fatty acids, lauric (C14) to stearic (C18), may be broken down by a specific long chain fatty acid peroxidase (Stumpf 1956). This peroxidase catalyzed the peroxidative decarboxylation of the fatty acid to yield carbon dioxide and an aldehyde with one less carbon atom. The aldehyde was then oxidized to its corresponding acid under the catalysis of an aldehyde dehydrogenase. Fatty acid peroxidase has been found in the cotyledons of germinating peanut and safflower seedlings, but was absent from lupine, soybean,

sunflower and castor beans. Stumpf (1956) described a specific long chain fatty acid peroxidase which was responsible for the partial oxidation of palmitic acid to carbon dioxide. Like iodid peroxidase, this peroxidase also required a peroxide generating system, and the direct addition of hydrogen peroxide did not enhance the reaction. The reaction was specific for fatty acid peroxidase; it could not be catalyzed by horseradish peroxidase together with a peroxide generating system.

Cytochrome c peroxidase, which has been isolated from Baker's yeast, is specific toward reduced cytochrome c. Unlike other peroxidases, including horseradish peroxidase, it was completely inactive toward pyrogallol, a general hydrogen donor for the peroxidase reaction. Since horseradish peroxidase was observed to be inactive toward cytochrome c, it was concluded that the two enzymes were different (Altschul, Abrams, and Hogness 1940). The molecular weight of cytochrome c peroxidase was determined to be 60,000 (Abrams, Altschul and Hogness 1942). Cytochrome c peroxidase is of interest because of its implications in cellular respiration.

### Peroxidase: Role in Plants

Location: Peroxidase was described by Saunders, Holmes-Siedle, and Stark (1964) as having "a degree of versatility not surpassed by any other single enzyme." In initial reactions it is somewhat specific, but in many cases, the primary oxidation product is itself capable of oxidizing or reacting with other substances present in the system which may not be oxidized directly by the peroxidase molecule. The number of reactions which could thus result from the primary reaction of peroxidase

are many, and it is clear why peroxidase is noted for its versatility. It is also obvious why so much difficulty has been encountered in trying to ascribe a function to plant peroxidase.

Peroxidase has been found in most plant tissues studied, with much variation in the amount of peroxidase activity among tissues. The root is commonly described as the organ with highest peroxidase activity (Lundegardh 1954; Goren and Goldschmidt 1966). Jensen (1955) studied the histochemical localization of peroxidase in roots and found that cells of the root cap, proepidermis, and vascular tissue showed peroxidase activity. Avers and Grimm (1959) found that in the root epidermis, peroxidase activity occurred in all growing cells, but was intensified in hair cell initials in the basal portion of the elongation zone.

Within a cell, peroxidase is believed to be localized in lysosomes or microbodies. Plesnicar, Bonner, and Storey (1967) found that in homogenates of mung bean hypocotyls, over 90% of the peroxidase was soluble, but a small amount remained membrane bound. Lanzani and Galante (1964) reported that wheat embryos contained a soluble, and a ribosome-linked peroxidase which could be solubilized by treating the ribosomes with EDTA. These two peroxidases had different kinetic properties.

Considerable literature exists on proposed functions for peroxidase in plants. In this literature review, only major roles for which good evidence exists will be described. Peroxidase will be examined in light of its role in the lignification process, growth regulation (through its action on auxin), and disease resistance mechanisms.

Peroxidase and Lignification: Lignin occurs only in plants, in secondary cell walls of woods, barks, stems, etc. In the past it was considered to be a chain-like polymer built up from a single simple unit, as are cellulose and starch. It is now known that lignin does not exist in situ as one individual component, but rather as a collection of similar molecules which are closely related structurally (Schubert 1965). Lignin has been defined as that wood constituent which, when oxidized with nitrobenzene, yields vanillin in the case of coniferous trees, vanillin and syringaldehyde in the case of deciduous woods, and p-hydroxybenzaldehyde, vanillin and syringaldehyde in the case of monocotyledons (Brauns and Brauns 1960).

Several enzymes have been implicated in the pathway of lignin synthesis. Phenolase, tyrosinase and laccase are among these, with laccase being the most likely. However, the distribution of laccase in higher plants is limited whereas that of peroxidase is not (Higuchi and Ito 1958), and peroxidase is capable of carrying out many of the same reactions as laccase. Freunderberg et al. (1951, 1952) found that the production of a lignin polymer from coniferyl alcohol was increased seven-fold by the addition of hydrogen peroxide, indicating the participation of a peroxidase.

Siegel (1953, 1954, 1955) determined that lignin could be formed when plant tissue sections were incubated with certain phenolic compounds such as eugenol and thymol in the presence of hydrogen peroxide. The reaction was also affected by a thermolabile, cyanide-sensitive catalyst. This all inferred that peroxidase was taking part in the reaction. It has also been found that the distribution of peroxidase

in tissues corresponds to the pattern of lignification (Siegel 1953). Similarly, Jensen (1955) reported that vascular tissue which was very high in peroxidase activity was active in lignin formation when provided with eugenol and hydrogen peroxide.

In direct contradiction to this, DeJong (1967) found that in onion root tissues, the peroxidase reaction was strongest in tissues which did not lignify and that peroxidase was totally absent from lignifying xylem through all stages of development. He concluded from this that plant peroxidase was not involved in lignification. However, there is no doubt that certain plant peroxidases are capable of carrying out the lignification reaction. NB

Peroxidase and Growth Regulation: In higher plants there is a system which causes the oxidative inactivation of the growth hormone indoleacetic acid (IAA). In 1935 Van Overbeek showed that corn tissues destroyed auxin by an enzymatic process, and furthermore, the auxin destroying power of the tissues was paralleled by their peroxidase activity. The enzymatic destruction of auxin was noted in other species also (Tang and Bonner 1947; 1948; Wagenknecht and Burris 1950) and the enzyme responsible was called IAA oxidase (Galston and Baker 1951).

Galston, Bonner and Baker (1953) found that the oxidation of IAA took place with the consumption of one molecule of oxygen for every molecule of IAA oxidized. Tang and Bonner (1947) determined that the enzyme was a heme protein, while Wagenknecht and Burris (1950) said that they had evidence that it was a copper containing protein. From a study of pea homogenates, Galston and Baker (1951) hypothesized that

IAA oxidase was a complex of a peroxidase and a flavoprotein. The flavoprotein was responsible for the production of hydrogen peroxide and the peroxidase then oxidized the IAA utilizing the released hydrogen peroxide. Goldacre, Galston, and Weintraub (1953) found that dichlorophenol greatly enhanced IAA oxidase activity in peas with the activity being either enhanced or depressed by various substitutions on the phenol ring. In 1953 Galston, Bonner, and Baker proved that in peas, the enzyme involved in IAA oxidation was peroxidase and in fact, crystalline horseradish peroxidase was also capable of oxidizing IAA.

Kenton (1955) demonstrated that waxpod bean root residue had a high peroxidase activity towards pyrogallol and that it would also oxidize IAA. Oxidation of IAA was increased in the presence of the manganous ion. In this case, the enzyme system was composed of two parts--a thermolabile fraction and a thermostable part; the thermolabile part could be replaced by horseradish peroxidase, but the thermostable fraction was not identified. He found that the IAA oxidase system of bean root did not depend on the presence of a flavoprotein as did that of peas. Stutz (1957) isolated IAA oxidase from Lupinus alba and found that it required a phenolic activator and was stimulated by the manganous ion.

Gortner and Kent (1953) determined that the IAA oxidase activity from pineapple leaf and meristems had somewhat different properties than other IAA oxidases. Pineapple peroxidase had a pH optimum of 3.5, in contrast to the optimum of 6.2 - 6.7 reported for peas (Tang and Bonner 1947) and 6.0 - 6.5 reported for beans (Wagenknecht and Burris 1950). It also required  $Mn^{++}$  for activity, but 2,4-dichlorophenol



gave no enhancement of the enzyme. Ray (1960) noted that IAA oxidase activity and peroxidase activity ran parallel in Omphalia flavida and concluded that they were due to a single enzyme. Shin and Nakamura (1962) isolated two peroxidases from wheat germ, each of which had IAA oxidase activity, but each had a different IAA oxidase activity. The two had different pH optima and were affected differently by the  $Mn^{++}$  ion.

Rice coleoptiles grown submerged in water rather than floating have shown a marked acceleration of growth (Wada 1961a). Homogenates of coleoptiles grown in water had a considerably higher IAA oxidase activity and it was determined that in rice coleoptiles grown under water an IAA oxidase inhibitor was present which decreased as the coleoptile was exposed to air (Wada 1961b). Wada suggested (1961c) that IAA oxidase and its inhibitor played a role in growth regulation in rice coleoptiles.

Siegel and Galston (1967a) reported that the peroxidase apoenzyme (enzyme with the heme removed) did not retain peroxidase activity but did however continue to show indoleacetic acid oxidase activity. Endo (1968) found that in root tissues of turnip, radish, sweet potato, spinach, and morning glory, about one half of the peroxidases, as separated by starch gel electrophoresis, also stained as indoleacetate isoenzymes. There have been numerous reports of peroxidase-catalyzed oxidation of IAA brought about in vitro by horseradish peroxidase (Klapper and Hackett 1963; Hinmann and Lang 1965; Fox, Purvis, and Nakada 1965; Fox 1967). Considering all reports, it appears that the functional part of the IAA oxidase system in plants is peroxidase, but the prosthetic groups and co-factors and optimum conditions vary

throughout the plant kingdom. This makes peroxidase a key enzyme in growth regulation.

Peroxidase and Disease Resistance: Following disease infection in plants, protein metabolism has been found to be altered, and is commonly accompanied by an increase in oxidative enzymes (Rudolph and Stahmann 1964; Tomiyama and Stahmann 1964). Barnett and McLaughlin (1954), using moving boundary electrophoresis, showed that a wheat variety susceptible to Puccinia triticini showed an altered protein pattern after infection, whereas a resistant variety did not. Uritani and Stahmann (1961) determined that in sweet potato, resistance to black rot was correlated with the ability of the plant to produce new protein after an initial infection. They identified one of these new proteins as a peroxidase.

Yu and Hampton (1964) found that following infection with Colletotrichum destructivum, tobacco leaf tissue had a higher peroxidase activity than uninfected tissue, and in addition, one new peroxidase isoenzyme was present. Staples and Stahmann (1963) and Staples (1964) found that when beans were infected with a rust fungus there was no change in the number of peroxidase isoenzymes. In contrast, Andreev and Shaw (1965) found that when flax was infected with rust, there was an increase in the number of peroxidase isoenzymes observed on acrylamide gel. Heat-killed disease organisms were observed to be capable of inducing an increase in host peroxidase activity (Lovrekovich, Lovrekovich and Stahmann 1968).

Peroxidase activity in both leaves and roots of virus-infected sweet potato plants was significantly higher than in healthy plants

(Loebenstein and Linsey 1961), and this increase in activity began with the appearance of the disease symptoms. Likewise, in tobacco plants infected with mosaic virus, peroxidase activity was found to be highest where symptoms were most severe (Loebenstein and Linsey 1966). It has been well documented that these observed changes in peroxidase patterns were determined by the host and not the infecting agent (Stavelly and Hanson 1967; Solymosy, Szirmai, Beczner, and Farkas 1967). Farkas and Stahmann (1966) found that young healthy beans had two peroxidase isoenzymes on acrylamide gel, and when infected with virus showed an additional two isoenzymes. The four peroxidases from infected tissues were isolated and purified and were found to be well defined proteins with different physicochemical and immunological properties.

Hadwiger and Schwochau (1969) proposed that the host resistance response in plants was due to a dominant host gene which was activated by specific substances produced by the pathogen. Once the gene was activated, it altered the host metabolism causing the production of an environment unfavorable to the pathogen. An increased peroxidase activity might contribute to such an environment.

## GENERAL METHODOLOGY

### Materials

Inbreds, genetic mutants, and tropical races of Zea mays L. were used from a collection of several hundred maintained at the University of Hawaii, Honolulu, Hawaii. The material was grown at the Hawaii Agricultural Experiment Station, either on the Manoa campus or at Waimanalo. Field grown materials were used as a source of developing and mature tissues, and also for the hand pollinations required for genetic crosses.

Seedling tissues were obtained from seeds germinated in petri dishes in the laboratory. The seeds were soaked in 5 percent chlorox for 20 to 30 minutes, rinsed with distilled water and allowed to stand in distilled water for 24 hours prior to germination on moistened filter paper in petri dishes. Root tissues were used 4-5 days after sowing, and other tissues were sampled at various times up to two weeks. Tissues were either used directly or were frozen at  $-4^{\circ}\text{C}$ . No differences in peroxidase patterns were generated by freezing.

### Methods

The method of horizontal starch gel electrophoresis was used for the separation of peroxidase isoenzymes. The procedure followed closely the original technique of Smithies (1955) as modified and described by Brewbaker, Upadhy, Makinen, and Macdonald (1968). The

following buffers were prepared in large quantities:

Buffer A (pH 8.1) 0.025 M lithium hydroxide (15.945 gm/ 15.2 liters water)  
0.200 M boric acid (187.994 gm/ 15.2 liters water)

Buffer B (pH 8.2) 0.010 M citric acid (29.119 gm/ 15.2 liters water)  
0.065 M Trizma base (119.639 gm/ 15.2 liters water)

A 13.3 percent starch gel was prepared in the following manner: Two hundred and seventy ml of Buffer B was mixed with 30 ml of Buffer A, and 200 ml of this solution was heated to boiling. Forty gm of hydrolyzed starch was suspended in the remaining 100 ml of buffer solution. The boiling 200 ml of buffer was then poured into the starch suspension, mixed well, and put under vacuum until all gas was removed. The starch was poured into a plexiglass tray (18 cm x 20 cm x 3 mm) and allowed to solidify at room temperature for approximately 20 minutes. Once the gel was firm and the excess starch at the edges trimmed off, it was ready for sample application.

Tissue extracts were prepared by macerating the tissue with a few drops of saline solution (0.8% sodium chloride, 0.1% sodium nitrate; pH 5.8) using a mortar and pestle. The extract was then taken up in a filter paper wick (5-10 mm x 3 mm). For pigmented tissues such as leaves, the extract was taken up in the wick through a small piece of lens paper. A slit was cut in the hardened gel 7 cm from one end, and the sample wicks inserted. As many as 20 samples could be applied to one gel.

The gel was connected by means of sponge bridges to buffer tanks containing enough Buffer A to cover the platinum electrodes. One end of the sponge dipped into the buffer while the other overlapped the gel approximately five cm on each end. This whole apparatus was

situated in the refrigerator at 4°C. The gel and the sponges were covered with polyethylene film (Saran wrap) and a glass plate was placed on top of this to equalize pressure on the sponges. The buffer in the tanks was changed after two runs. The tanks were hooked up to a DC power source, and a current not exceeding 50 ma per gel was applied across the gel. After 4 to 5 hours the isoenzymes had separated sufficiently and a borate front was visible approximately 7 cm from the origin.

When the electrophoretic run was completed, the gel tray was removed from the buffer tank assembly and prepared for staining. Starch beyond the borate front was cut off and discarded, since no isoenzymes preceded the front. The gel was sliced horizontally with a cheese cutter, and the bottom portion used to stain for peroxidase isoenzymes. In some cases both top and bottom slices were placed in stain solution.

The peroxidase stain solution consisted of a suitable hydrogen donor and hydrogen peroxide. The principle hydrogen donor used in this study was o-dianisidine (3,3'-dimethoxybenzidine), following closely the stain solution as outlined by Owen, Silberman, and Got (1958). Approximately 150 mg of o-dianisidine was dissolved in 100 ml of 95% ethanol. Fifteen ml of acetate buffer at pH 4.7 (0.88 M sodium acetate and 0.62 M glacial acetic acid) and 25 ml of water were added. Immediately before staining, 3.5 ml of 3% hydrogen peroxide was added. This stain solution was poured over the gel and allowed to stand at room temperature until pinkish-brown bands appeared, indicating zones of peroxidase activity. The time required for intense staining ranged from 15 to 20 minutes, depending on the tissues and isoenzymes studied. When staining was complete, the solution was poured off and the gel

rinsed with water. Data were either recorded off the gel immediately, or the gel was saved for photography and/or drawing. If the gel was to be saved, it was left in water overnight and then soaked in 70% ethanol for 15 to 30 minutes. This treatment dehydrated the gel, making it firmer and easier to handle, with very little loss in band resolution due to increased opacity of the gel. The gel was blotted dry, wrapped in Saran and stored in the refrigerator. The longevity of gels treated in this manner extended to periods up to three years.

Benzidine and guaiacol were also tested as hydrogen donors. Benzidine stain was prepared by heating 1 gm of benzidine in 9 ml of glacial acetic acid until it dissolved. Forty ml of water was added, and then equal parts of this solution and 3% hydrogen peroxide were mixed and poured over the gel. Almost immediately the areas of peroxidase activity stained bright blue. The stain was then poured off and the gel rinsed with water. The reaction products of benzidine were not stable and bands soon faded. Consequently, benzidine-treated gels could not be saved and had to be photographed for a permanent record.

Guaiacol-containing stains were prepared by mixing 5 to 7 drops of guaiacol in 100 ml of 0.2 M phosphate buffer at pH 6.2. The gel was soaked in this solution for 15 to 20 minutes and then 10 ml of 3% hydrogen peroxide was added. With guaiacol, the peroxidase isoenzymes stained purple in about 10 minutes. The staining solution was then poured off and the gel rinsed with water. The gel was soaked in glycerine to preserve the bands and harden the gel. A glycerine-treated gel was somewhat translucent and could be stored in the refrigerator for long periods of time.

## RESULTS AND DISCUSSION

### Substrate Specificities

Three hydrogen donors, benzidine, o-dianisidine, and guaiacol were examined at the beginning of this study. Benzidine gave excellent staining, but the bands faded rapidly and the gels could not be preserved easily. Factors affecting the benzidine, hydrogen peroxide, peroxidase reaction and the stability of the reaction products were investigated by Straus (1964). O-dianisidine and benzidine produced identical zymograms however, and since o-dianisidine reaction products were stable and provided a permanent stain, o-dianisidine was selected in preference to benzidine for most studies. Related studies in this laboratory have compared benzidine and o-dianisidine on other plant tissues with similar results.

Guaiacol did not stain all of the isoenzymes of maize which appeared with o-dianisidine or benzidine. The faster moving anodal isoenzymes were never seen when guaiacol was used as hydrogen donor. It was concluded that certain maize peroxidases behaved differently toward the two hydrogen donors, o-dianisidine and guaiacol, when used under optimal conditions (pH 4.7 for o-dianisidine and pH 6.2 for guaiacol). Macko and Novacky (1966) also observed that maize peroxidases reacted differently to benzidine, guaiacol and catechol; Lushchinskaya and Ermakov (1968) also found that peroxidase isoenzymes in corn behaved differently with benzidine, guaiacol, and pyrogallol. It was observed in a study of peroxidase in papaya fruits (Hamill unpubl.) that although both o-dianisidine and guaiacol indicated peroxidase activity as measured colorimetrically,



only o-dianisidine would stain zones of peroxidase activity on a starch gel. These results serve to point out the hazards of comparing reports of peroxidase isoenzymes or of drawing analogies between peroxidase isoenzyme patterns and peroxidase activity measured spectrophotometrically, if the same hydrogen donor was not used.

All data reported in this thesis were taken from zymograms where o-dianisidine was used as the hydrogen donor. These isoenzymes also showed some activity with pyrogallol and the iodide ion, but clear resolution was never obtained; none of them showed catechol oxidase activity.

#### Peroxidase Isoenzyme Variation in Maize Tissues

Isoenzyme variation was studied in detail in several tissues of maize inbred AA8 at several stages of development to assess the extent of tissue variation of peroxidase isoenzymes in maize (Figure 1). A composite of all tissues revealed 21 stained regions in this inbred, 12 migrating to the anode and 9 migrating toward the cathode at pH 8.1 used. In addition, certain tissues, e.g. root, leaf, and pollen were studied in many different genotypes and will be discussed later.

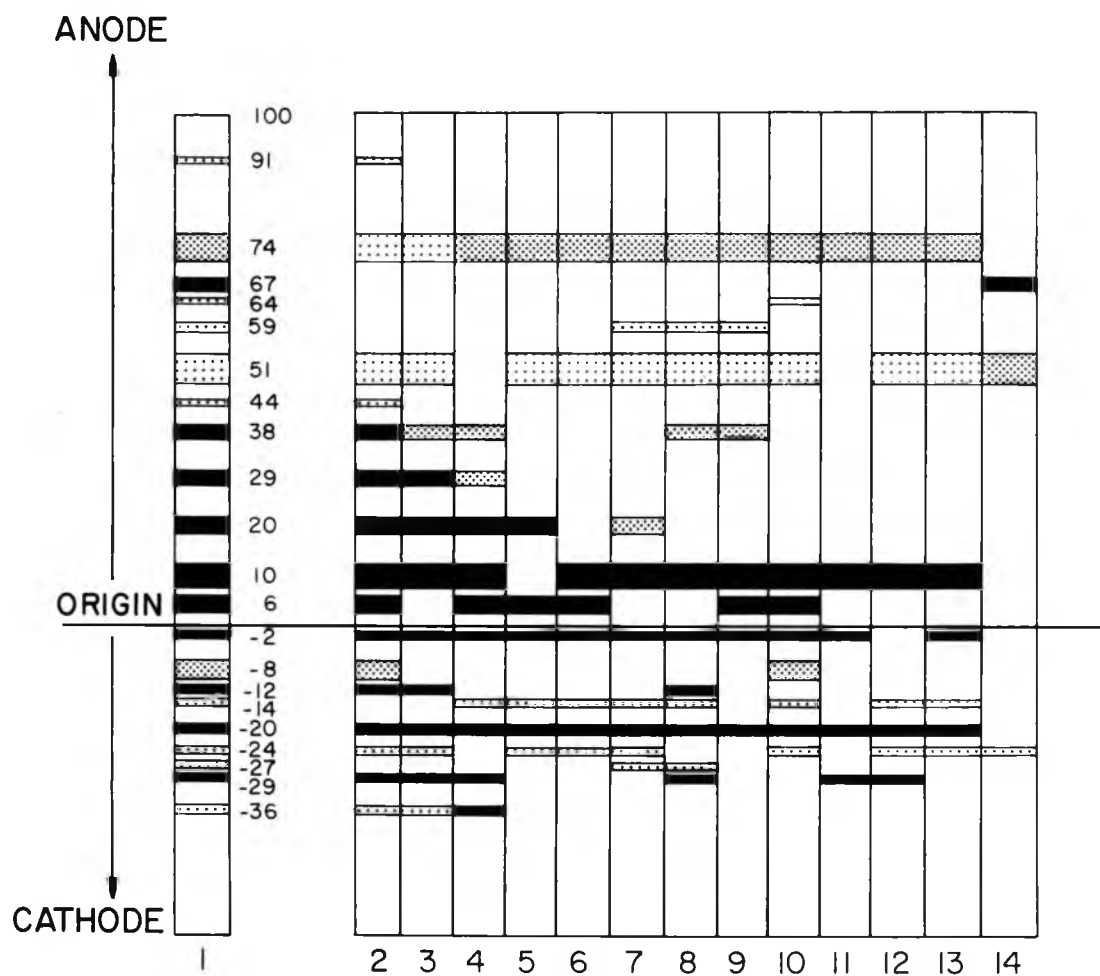
Tissue samples of approximately 0.05 gm wet weight were macerated in a saline solution and subjected to starch gel electrophoresis (see Materials and Methods). Pollen required a somewhat larger sample, approximately 0.5 gm. Position of bands was calculated as a percentage relative to the borate front. The relative positions of cathodal isoenzymes were presented as negative values. For convenience, these relative movement values have been used to designate the bands. The values were found to be highly repeatable, providing that care was taken to place the cathodal sponge at the same position from the origin on each

gel. The coefficients of variation for relative movement values of bands 74 and -20 were calculated and found to be 6.3% and 14.3% respectively. The intensity of shading in Figure 1 indicates approximately the intensity of o-dianisidine staining on the gel.

A seedling root of inbred AA8 was chosen as a standard to be run on each gel since seedling roots had a good portion of the total isoenzyme complement of maize. Bands from different tissues were considered to be the same isoenzyme if they ran to the same spot when placed side by side on the starch gel. This was easier to determine on the anodal side of the gel than on the cathodal side since anodal isoenzymes were more widely separated. Isoenzymes -8, -12, and -14 and isoenzymes -24, -27, and -29 (Figure 1) were close together in the system used here and sometimes did not stain darkly. The relative movements of cathodal peroxidases were also affected by the distance of placement of the sponge from the origin, making it difficult to definitely establish identity of these bands on some gels. However, there was no doubt about the identification of the other isoenzymes. In Figure 1, 21 bands are shown for inbred AA8. More bands than this were observed in all maize stocks studied since several of these isoenzymes proved to be genetically polymorphic.

A brief description of isoenzymes diagrammed in Figure 1 follows. Isoenzyme 91, the fastest moving anodal band, stained faintly and was observed only in leaf blade tissues of young (ca. 30 day old) plants. It was never seen in any other tissues and was not consistently present in the leaf blade. Isoenzyme 74 was a medium staining isoenzyme seen in all tissues at some time in their development. The only place that it was consistently seen however, was in the root tissues. This isoenzyme

Fig. 1. The Peroxidase Isoenzymes of Maize Inbred AA8.  
(1) Composite of all Isoenzymes. (2) Leaf Blade.  
(3) Leaf Sheath. (4) Root. (5) Stalk Prior to  
Elongation. (6) Internode. (7) Tassel Stalk.  
(8) Tassel Glume. (9) Anther. (10) Husk. (11) Silk.  
(12) Kernel. (13) Cob Pith. (14) Pollen.



sometimes appeared to resolve into two indistinct bands, but was most often observed only as a single band. A very rapidly staining dark band which occurred in mature pollen only was designated isoenzyme 67. Pollen of certain inbreds was observed to have a slower moving variant of this band, moving to position 62. Isoenzyme 64 was a poorly resolved band seen only in husk material. Isoenzyme 59 stained faintly but clearly, and was present in developing tassel material, in the tassel stalk, glumes, and anthers. It was not seen in any other tissues nor in mature tassel tissues. At position 51 there was a wide area which stained faint to medium. It never resolved into a crisp (sharply defined) isoenzyme with the techniques used here, and was seen in most tissues at some time during development. Isoenzyme 44 was another faint staining doubtful band which was seen only in the leaf blade during development. Isoenzymes 38, 29, 20, and 10 were all sharp bands observed most clearly in leaf blade and sheath and older root material. Studies revealed these to be a series of related bands and these will be discussed later. When many inbreds were surveyed, it was found that two patterns existed, one with bands having relative movements as described here, and one displaced slightly towards the anode. Isoenzyme 6 was a densely staining band, present in many tissues and was often contiguous on the gel with isoenzyme 10, giving the appearance of a single wide band.

Isoenzyme -2 was universally present in all tissues with the exception of kernels. Isoenzyme -8 was a medium staining band seen only in leaf blade and husk material. Isoenzymes -12 and -14 were very close; -12 was found in blade, sheath and anthers while -14 was observed in many tissues. Isoenzyme -20 was the only isoenzyme which was found to be

present in all tissues. Isoenzyme -24 was an inconsistently staining band. Isoenzyme -27 stained faintly and was observed only in tassel material, glumes and stalk. Isoenzyme -29 stained clear and dark, and occurred in many tissues. It was studied in much detail in seedling root tissues and it was found that there were two variants of this isoenzyme, one a little faster and one a little slower. Isoenzyme -36 was seen in leaf blade and sheath. It ran in front of isoenzyme -29 and often was not distinguishable as a band, but rather as a shadow.

The amount of tissue isoenzyme specificity seen in maize peroxidases was consistent with that observed in other species. Scandalios (1964) and Macdonald and Brewbaker (Unpubl.) also noted tissue specific isoenzymes for maize esterases, leucine aminopeptidases, peroxidases, and catalases. Siegel and Galston (1967b) observed characteristic peroxidase isoenzyme patterns for different organs in pea seedlings, and Tyson and Jui (1967) found that in Linum usitatissimum there were consistent differences in peroxidase activities among genotypes and among tissues.

For descriptive purposes, the life of the maize plant has been divided into 11 stages following Hanway (1963). Certain corn tissues were sampled at 6 of these stages and observed for peroxidase isoenzymes to assess the magnitude of isoenzyme variation with development. The stages as described by Hanway were:

- Stage 1. Collar of 4th leaf visible
- Stage 2. Collar of 8th leaf visible
- Stage 3. Collar of 12th leaf visible
- Stage 4. Collar of 16th leaf visible. Tassel visible
- Stage 5. 75% of the plants have silks visible
- Stage 6. 24 days after 75% silk

Isoenzyme patterns changed as all tissues developed. Roots showed a decrease in peroxidase isoenzymes during maturation. In stages 1 and 2, roots exhibited 9 clear isoenzymes (74, 20, 10, 6, -14, -20, -29, and -36). By stage 3, only 4 bands remained (74, 10, 6, and -36) and this pattern did not change after Stage 3.

In contrast to root tissues, the leaf material increases in complexity as the plants matured. At any one stage, it was found that a greater number of peroxidase isoenzymes was present in the lower leaves, i.e. the older leaves. At Stage 1, there were five leaves which were analyzed. The fifth or youngest leaf blade had six isoenzymes (51, 20, 10, -2, -20, -29) whereas the first leaf blade had ten isoenzymes (51, 38, 29, 20, 10, -8, -20, -24, -29, and -36). The most striking change was the gradual development of the darkly staining series of anodal isoenzymes 10, 20, 29, 38, and 51. Whether leaves of different ages were compared on a single plant or on two plants of different ages, this increase in complexity with age was noted. As long as the leaf was growing, the full complement of isoenzymes was not seen. However, as soon as the leaf was fully elongated and the total number of peroxidases seen, this pattern remained constant until the leaf died. The isoenzymes of leaf sheath followed closely those of the blade in developmental sequence. There was a time lag however, with the leaf sheath increasing in complexity of pattern less rapidly than the corresponding leaf blade.

In internode tissue, a change in peroxidase pattern appeared to be correlated with the cessation of elongation. This was clearly demonstrated in Stage 3 material, in which the lowest five internodes were fully elongated. In these five internodes, only three isoenzymes (10, -14, and -20) were observed, of which isoenzyme 10 stained

intensely, while -14, and -20 were faint. The next four internodes had not completed elongation; they too had three isoenzymes, but the pattern was different, with isoenzymes -14 and -20 staining strongly and isoenzyme 51 staining faintly. There was a definite shift during internode development from strongly staining cathodal peroxidase isoenzymes to strongly staining anodal isoenzymes, which appears to be correlated with the completion of the elongation process.

The role of plant peroxidase in relation to auxin metabolism and growth regulation has been discussed previously (literature review). Auxin has been noted to cause repression of peroxidase isoenzymes (Sarkissian and Spelsberg 1967; Ockerse, Siegel, and Galston 1966) and also to promote the synthesis of peroxidase (Lavee and Galston 1968). Auxins contribute to the control of tissue elongation in plants (Leopold 1964), and it appears that peroxidase may be important in determining growth by acting as indoleacetic acid oxidase on the growth hormone. No satisfactory stain solution specific for indoleacetic acid oxidase was developed in this study, although a gel technique has been described by Endo (1968). It appears that isoenzymes must be removed from the gel and assayed for IAA oxidase activity by means of a colorimetric test (Meudt and Gaines 1967).

IAA oxidase activity of the peroxidase isoenzymes described here remains to be determined. However, it might be suggested that the peroxidase isoenzymes which developed in association with the elongation process, as for example, bands 10, 20, 29, 38, and 51 in leaf material and band 10 in internode, are acting principally in the capacity of IAA oxidases, bringing about the cessation of auxin activity and hence the cessation of elongation. This idea is supported somewhat by the fact that in the



leaf sheath these isoenzymes appear a little later in time than they do in their corresponding blade. Leaf sheath and internode elongation continues for some time after the leaf blade is fully elongated. At Stage 3, leaves have essentially attained full area although they are still enclosed in the whorl (Hanway 1963). Leaf sheath must continue to elongate along with the internode until the plant has reached its final height at Stage 5. The leaf peroxidases 10, 20, 29, and 38 always developed as the leaf blade or sheath was completing elongation. These isoenzymes are members of a related series controlled by a single locus,  $Px_3$ , to be described later, and appear to be probable auxin oxidases.

Immature anthers were first analyzed at Stage 3, with high anodal peroxidase activity (bands 74, 59, 51, 38, 6, and -20). At Stage 4, in mature anthers prior to anthesis but subsequent to meiosis, only two bands, 6 and -20 were observed. At Stage 5 (anthesis) the pattern was again different, with bands 74, 51, and -20 being present and darkly staining. Mature pollen displayed isoenzymes 67, 51, and -20.

Isoenzyme changes appeared to be intimately involved with the metabolic activity of a tissue. At Stage 3, the pollen mother cells are undergoing meiosis and cells are actively synthesizing and dividing. At Stage 4, pollen cells have been formed but the tassel is still in the whorl and it will be some time before it must carry out fertilization; it might be assumed that at this time there is little metabolic activity within the anthers. At Stage 5 the anthers were just about to shed their pollen, and the bands stained very intensely. Band 67 was observed to be exclusively a pollen isoenzyme, found only in mature pollen which had been shed.

No attempt was made in this study to correlate peroxidase activity or isoenzymes with a particular stage in pollen development. Linskens (1966) using disc electrophoresis, found that during pollen meiosis and development in petunia, each cytological stage showed a specific protein pattern. Isoenzymes were found in various numbers and activities, especially the peroxidases and the lactate dehydrogenases. The observations noted here with maize are in agreement with other work.

The abundance of peroxidase isoenzyme variation which was observed in the tissues of maize provided material for genetic analysis of several of the isoenzymes. Five loci will be documented in the following sections of this dissertation.

#### Genetic Polymorphisms

Following a survey of peroxidase isoenzyme polymorphisms in maize tissues, five polymorphisms were selected for further genetic study. They were designated as follows:  $Px_1$  studied in root tissues,  $Px_2$  studied in pollen,  $Px_3$  studied in mature leaves and seedling root, and  $Px_4$  and  $Px_5$  studied in mature leaves. The nomenclature used here is "Px" to indicate a peroxidase locus; a subscript distinguishes loci in order of their discovery; and a superscript indicates different alleles of one locus.

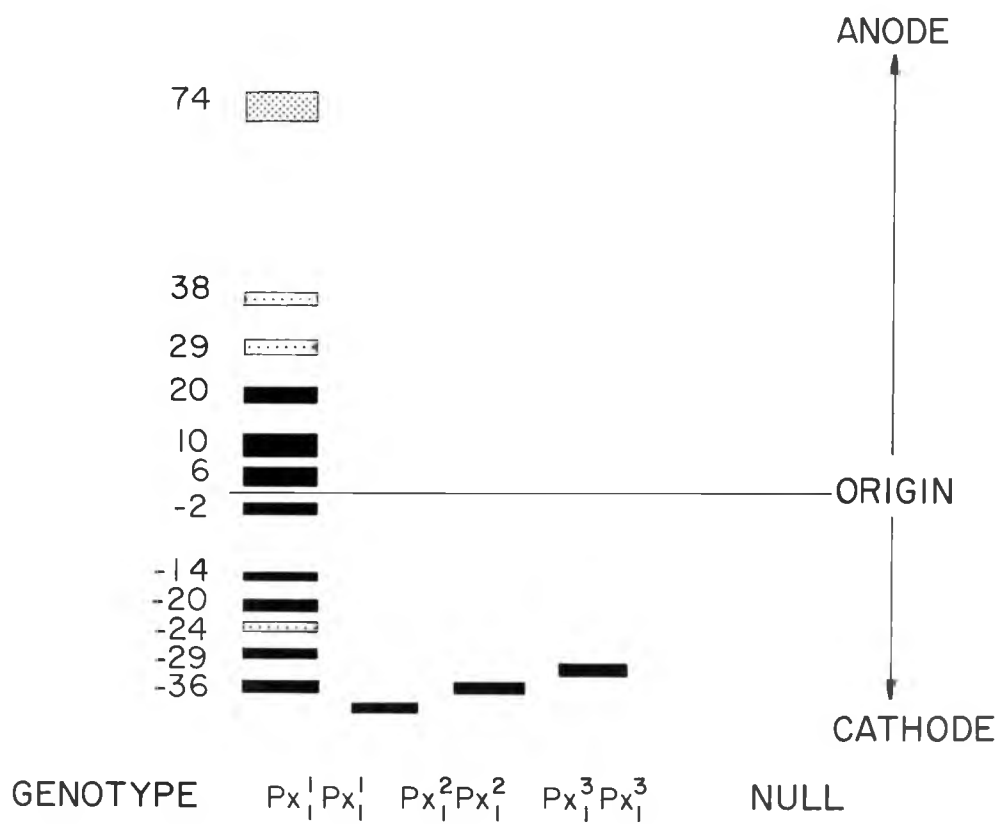
$Px_1$  Locus:  $Px_1$  was the designation given to a locus controlling a peroxidase isoenzyme system which moved cathodally to position -36 in the pH 8.1 buffer system used. It was first observed in seedling root and later was seen also in mature roots. Bands which moved to the same region on the

starch gel were also found in some leafy tissues. Positive identity of bands can be established by isolation, purification and chemical analysis of the isoenzymes, but identity may be inferred if the presence of the band in one tissue is consistently correlated with its presence in another, and if the inheritance in both tissues proves to be the same.

In the case of the  $Px_1$  peroxidases, the root isoenzyme was very clear and consistent throughout the life of the root, from the time of germination until the plant was mature, while in the leaf, the band at this position was somewhat variable and very inconsistent. In young seedling leaves in some lines, it was evident that the  $Px_1$  band was correlated in root and leaf; in other lines however, this was not so clear and the identification became more nebulous as the leaf matured. For these reasons no genetic data were recorded from leaf material, and if these two isoenzyme systems are identical, their action appears to be somewhat different in the two tissues. Perhaps leaf material contains modifiers or regulators serving to suppress expression of this isoenzyme whereas the root does not.

Seedling root samples of many maize lines were screened for polymorphism of the isoenzyme. Three variants were found, each moving to a slightly different position on the starch gel. In addition, there was a null condition, in which none of the variants was present. The three bands were given numbers 1, 2, and 3, with number 2 being the isoenzyme located at position 36, number 1 slightly ahead (that is, more positively charged) and number 3 slightly behind 2 (Figure 2). A study of diverse tropical racial material and genetic stocks revealed that these four isoenzyme patterns were all present in natural open-pollinated

Fig. 2.  $Px_1$  Patterns in Seedling Root of Maize.  
At Left is the Total Peroxidase Isoenzyme  
in Maize Root.



populations, but no attempt was made here to determine allelic frequencies. Inbred lines typically exhibited only one of the  $Px_1$  bands i.e. two of the isoenzymes were never found fixed together in any one inbred. This strongly indicated that the four variants might comprise an allelic system.

To test for allelism, the four variants were crossed by hand in all possible combinations to produce the  $F_1$ 's, and in the next generation,  $F_2$  and backcross progenies were made for each cross. Inbreds used for these crosses were AA3, H49, AA2, L289, A295 and M14. In all cases it was found that these inbreds possessed only one of the  $Px_1$  bands and the  $F_1$  of a cross between any two of these showed both parental bands; hybrid bands were never seen. Several hundred of these parental and  $F_1$  crosses were analyzed on the starch gel and no deviations from the expected phenotypes were observed. The data from the  $F_2$  and backcross populations are summarized in Table 1, where it may be seen from the Chi square values that the variants behaved as if the three bands and the null were each due to an allele of a single locus. The locus was designated  $Px_1$  with alleles  $Px_1^1$ ,  $Px_1^2$ ,  $Px_1^3$ , and  $Px_1^{null}$  (alleles 1, 2, and 3 are responsible for bands 1, 2, and 3 respectively). The genotypes of each of the inbreds used in this analysis with respect to the  $Px_1$  locus were thus determined to be as follows:

<u>Inbreds</u>	<u>Genotype</u>
AA3; H49	$Px_1^1 Px_1^1$
AA2; L289	$Px_1^2 Px_1^2$
A295	$Px_1^3 Px_1^3$
M14	$Px_1^{null} Px_1^{null}$

Table 1. Observed F<sub>2</sub> and Backcross Ratios for Crosses Involving Four Alleles at the Px<sub>1</sub> Locus in Maize

Parentage	Number of Progenies in each Phenotypic Class							Expected Ratio	Total	χ <sup>2</sup>	P
	1	2	3	0	12	13	23				
(AA3xAA2)x(AA3xAA2)	32	42	--	--	62	--	--	1:1:2	136	2.529	0.25-0.50
(AA3xAA2)xAA3	48	--	--	--	55	--	--	1:1	103	0.476	0.25-0.50
(AA3xAA2)xAA2	--	32	--	--	39	--	--	1:1	71	0.690	0.25-0.50
(H49xA295)x(H49xA295)	24	--	26	--	--	46	--	1:1:2	96	0.250	0.75-0.90
(H49xA295)xH49	21	--	--	--	--	26	--	1:1	47	0.532	0.25-0.50
(H49xA295)xA295	--	--	17	--	--	13	--	1:1	30	0.533	0.25-0.50
(H49xM14)x(H49xM14)	60	--	--	16	--	--	--	3:1	76	0.631	0.25-0.50
(H49xM14)xH49	33	--	--	--	--	--	--	1:0	33	--	--
(H49xM14)xM14	33	--	--	31	--	--	--	1:1	64	0.063	0.75-0.90
(L289xA295)x(L289xA295)	--	21	27	--	--	--	43	1:1:2	91	1.063	0.50-0.75
(L289xA295)xL289	--	23	--	--	--	--	17	1:1	40	0.900	0.25-0.50
(L289xA295)xA295	--	--	29	--	--	--	31	1:1	60	0.067	0.75-0.90
(L289xM14)x(L289xM14)	--	72	--	25	--	--	--	3:1	97	0.031	0.75-0.90
(L289xM14)xL289	--	76	--	--	--	--	--	1:0	76	--	--
(L289xM14)xM14	--	29	--	29	--	--	--	1:1	58	0.000	0.90-0.99
(A295xM14)x(A295xM14)	--	--	45	14	--	--	--	3:1	59	0.051	0.75-0.90
(A295xM14)xA295	--	--	60	--	--	--	--	1:0	60	--	--
(A295xM14)xM14	--	--	30	30	--	--	--	1:1	60	0.000	0.90-0.99

In the course of this study, a few other crosses were observed for their segregations at the  $Px_1$  locus. Crosses involving inbreds with the same  $Px_1$  allele showed no segregation in  $F_2$  and backcross populations. The  $F_2$  of the cross AA3sh ( $Px_1^1$ ) x 853 ( $Px_1^2$ ) gave a ratio of 5 (band 1): 16 (bands 1 and 2): 8 (band 2) fitting the 1:2:1 ratio and adding support to the idea that bands 1 and 2 are governed by alleles at one locus.

In Table 2, 121 inbreds have been listed in their appropriate  $Px_1$  class. Allele number 2 is by far the most common, being found in 67% of the field and sweet corn inbreds studied. Allele number 1 was seen in 17%, number 3 was observed in 11% and the null was found in only 5%. This represents a rather limited sample of corn inbred lines, and it was not determined if such differences in allelic frequencies also existed in open-pollinated populations.

An attempt was made to localize the  $Px_1$  locus on its chromosome using a series of B translocation stocks obtained from the Maize Genetics Cooperative. These stocks each have a piece of a B chromosome translocated onto one of the normal chromosomes and can provide information on the location of a gene by causing aberrant ratios to be produced in the  $F_1$ . This series of translocations was first described and used by Roman (1948) and is still incomplete, since there are translocations available for only seven of the twenty arms of the ten maize chromosomes. The B translocations available are B translocations with the long arm of chromosome 1 (1a), the short arm of chromosome 1 (1b), the long arm of chromosome 3 (3a), the long arm of chromosome 4 (4a), the short arm of chromosome 7 (7b) the short arm of chromosome 9 (9b), and the long arm of chromosome



Table 2.  $Px_1$  Alleles of 121 Maize Inbreds

Class	Inbred
$Px_1^{null}$	442, A239, C123, M14, P8, P39
$Px_1^1$ F	380, A297, A545, AA3, AA3sh, AA12, AA16, AA17, AA18, AA20, AA21, H49, H52, Mo5NR, N6D, Oh45, R53, R75, R117, R168, Wf9
$Px_1^2$ M	190a, 303b, 395a, 382, 442, 647, 650, 661, 727, 5446, 5480, 5490, A257, AA1, AA2, AA4, AA5, AA6, AA7, AA8, AA9, AA10, AA11, AA13, AA14, AA2sh, AA4sh, AA5sh, AA7sh, AA8sh, AA9sh, B8, B10, B37, B37TRF, B46, C42, C53, C114, C121, Hy2, I153, K201, L289, L304A, MVR1, MVR2, MVR3, MVR4, MVR5, MVR6, N24, Oh7B, Oh28, P51, R2, R84, R78, R101, R109B, R151, R839, R851, R853, R902, R917, S8, T8, T19, T20, T23, T24, T25, T35, T36, T47, T55, Tx203, Va17, Va35, W153R
$Px_1^3$ S	426, 541, 713, 2253, A295, B190, C102, C114, M119, R172, S4, T21, T54

10 (10a).

Seeds of the translocation stocks were germinated in Petri dishes, roots were sampled and analyzed for the  $Px_1$  allele, and were then transplanted to the field where hand pollinations were carried out. Translocation stocks were crossed as male onto a female peroxidase tester carrying a different  $Px_1$  allele. In addition, translocation stocks were crossed onto a suitable tester to verify that it was carrying the translocation (Roman 1948).  $F_1$  seeds of the peroxidase tester x B translocation cross were germinated in Petri dishes and root samples were analyzed on starch gel for their  $Px_1$  patterns. If the  $Px_1$  locus was on the chromosome involved in the translocation, then it was expected that some seedlings would show the phenotype of the female parent only, rather than the  $Px_1$  alleles of both parents.

It was later determined that some of the translocation stocks were carrying the null allele when they had been previously believed to be homozygous for another allele. This gave misleading results for the data from translocations 1a and 1b, and thus they have been omitted from this discussion until they can be further checked. None of the crosses produced here gave rise to offspring with a phenotype of the female only (Table 3). This series of crosses served to eliminate chromosome arms 3a, 4a, 7b, 9b, and 10a as the site of  $Px_1$ , and a positive identification of location may now be made using other means such as the waxy translocations.

Table 3. Phenotypes of  $F_1$  Progenies from Crosses Involving  
B Chromosome Translocations (male) and  $Px_1$  Testers (female)

Cross	Number of $F_1$ seedlings showing phenotype of both parents	Number of $F_1$ seedlings showing phenotype of female only
$Px_1$ x Transl. 3a	91	0
$Px_1$ x Transl. 4a	15	0
$Px_1$ x Transl. 7b	24	0
$Px_1$ x Transl. 9b	104	0
$Px_1$ x Transl. 10a	108	0

Px<sub>2</sub> Locus: Pollen presented a deeply-staining, unique peroxidase isoenzyme pattern, and some problems with separation and staining were encountered with pollen which were not found with other tissues. It was determined that about 0.5 gm of pollen in 2-3 drops of saline solution provided the clearest resolution. Pollen seemed to have a high amylase activity, and if the concentration of pollen was too great the starch gel was digested at the origin so that peroxidases were not satisfactorily separated and discernible. However, using approximately 0.5 gm of pollen in 2-3 drops of saline and running the gel until the front had moved about 5 1/2 cm, repeatable results were obtained.

In over 50 lines studied, the cathodal side of the gels showed only a single light staining band in position -24 and no polymorphism was seen for this isoenzyme. On the anodal side of the gel there was one densely staining zone of activity (position 51) which was universally present in all pollen samples examined. At position 67 was a zone which stained very densely and very rapidly, much more so than any other isoenzymes in any other tissues (Figure 1). In immature pollen (prior to anthesis) isoenzyme 74 was also seen. If pollen was collected immediately following anthesis this isoenzyme was still observed. However, after one hour of storage, either at room temperature or under refrigeration, this band did not appear. Between bands 51 and 67 a faint staining band occasionally appeared immediately in the stain and faded rapidly when the stain was removed. The other bands (-24, 51, 67) were consistent entities and were not affected by refrigerated storage over long periods of time. Although these pollen peroxidases behaved somewhat differently than peroxidases in other tissues, primarily in their intensity and rapidity of staining, they were considered to be peroxidase isoenzymes

according to the definition stated in the introduction of this thesis.

When several inbreds were examined, it was found that there was a variant for isoenzyme 67 which was somewhat slower moving and migrated to a position of 62 (Figure 3). For this peroxidase system, designated  $Px_2$ , the faster band (67) was given the number 1 and the slower band (62) was called band 2. In Table 4 are listed several inbreds and their  $Px_2$  class. Band number 2 was observed more frequently and was seen in all of the Hawaiian inbreds (AA's) and about half of the other Corn Belt lines studied. These two polymorphisms were the only 2 variants seen for the  $Px_2$  peroxidases, but the sample was rather small and it is highly probable that more variants do exist.

The  $F_1$  was made between these two variants using L289 (band 1) and M14 (band 2), and in the following generation the  $F_2$  backcross progenies were produced and freshly-dehiscing tassels used for pollen samples. The  $F_1$  showed both parental bands, and in the  $F_2$  and backcrosses, ratios were obtained indicating that these two bands were governed by a single locus (Table 5). This locus was designated  $Px_2$  with alleles  $Px_2^1$  and  $Px_2^2$ . L289 is thus of genotype  $Px_2^1 Px_2^1$  and M14 has genotype  $Px_2^2 Px_2^2$ .

Peroxidase has been taken as an indication of pollen viability for some species (King 1960). Maize is among that group of plants where pollen is shed in the trinucleate state, and these plants typically lose pollen viability rapidly with storage (Brewbaker 1967). Pollen studied here did not lose all of its peroxidase activity with loss of viability since isoenzymes -24, 51, and 67 were still present after long periods of storage (freezing), at which time the pollen was incapable of

Fig. 3.  $Px_2$  Patterns in Maize Pollen.  
At Left is a Complete Pollen  
Pattern in Maize Root.

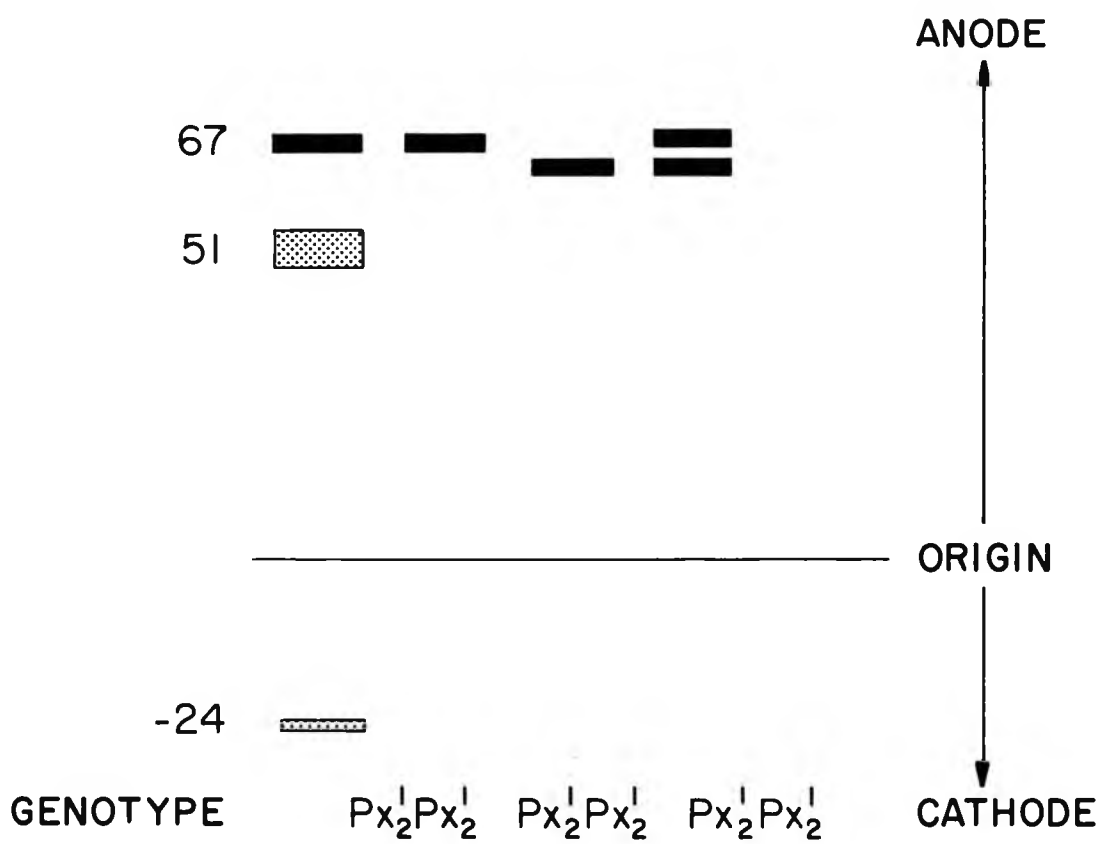


Table 4.  $Px_2$  Alleles of 49 Maize Inbreds

Class	Inbred
$Px_2^1$ (Fast)	442, 647, A295, AA1, AA2, AA3, AA4, AA5, AA6, AA7, AA8, AA9, AA10, AA11, AA12, AA13, AA14, AA15, AA16, AA17, AA18, AA19, AA20, AA21, AA22, AA23, B37, B37TRF, CI66, CM104, F6, GE54, H49, H52, Hy2, L289, N28, Oh43, T115, Wf9
$Px_2^2$ (slow)	38-11, 661, C42, C53, B14, C103, M14, M14NR, P39

Table 5. Observed  $F_2$  and Backcross Ratios for Crosses Involving Two Alleles at the  $Px_2$  Locus in Maize Pollen

Parentage	Number of Progenies in each Phenotypic Class			Expected Ratio	Total	$\chi^2$	P
	1	2	12				
(M14xL289)x(M14xL289)	17	20	41	1:1:2	88	1.523	0.25-0.50
(M14xL289)xL289	29	--	33	1:1	62	0.258	0.50-0.75
(M14xL289)xM14	--	33	29	1:1	62	0.258	0.50-0.75

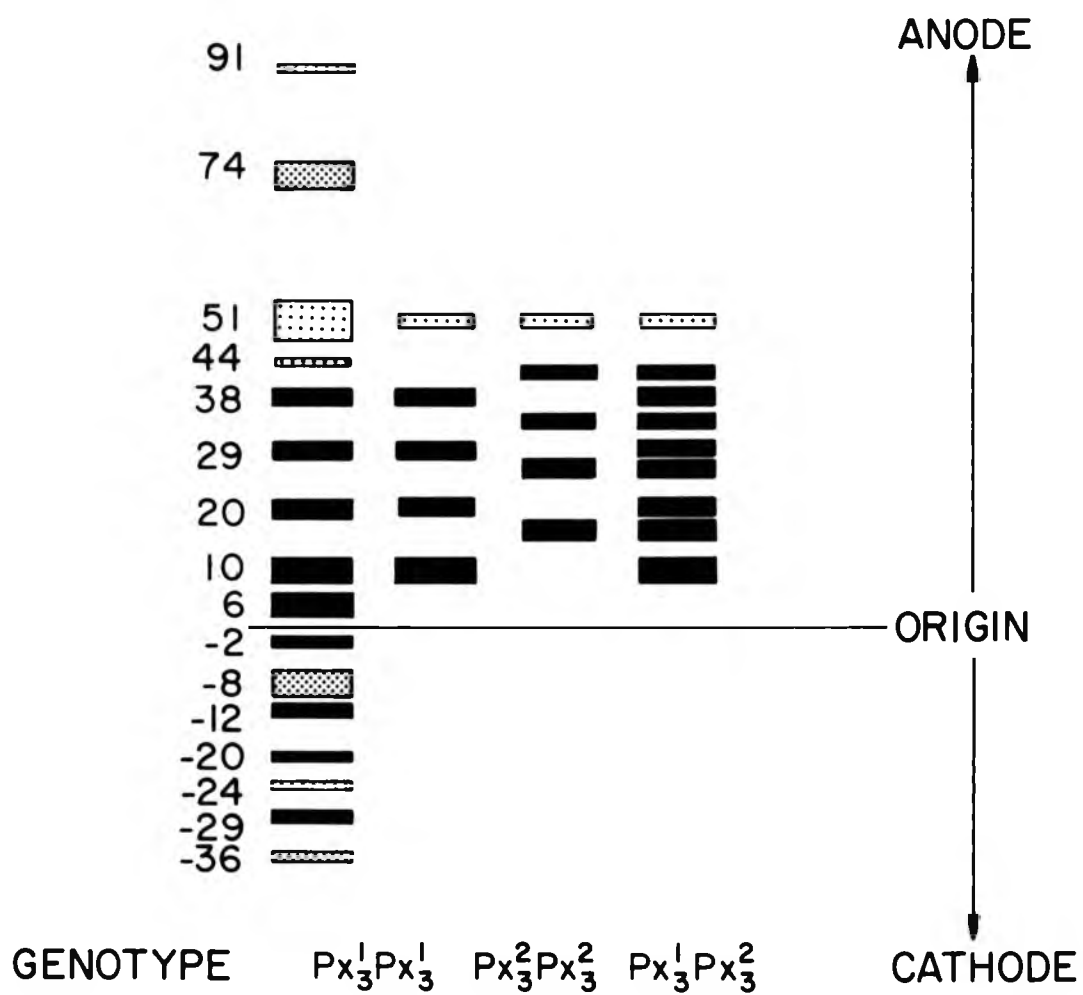


carrying out fertilization. On the other hand, the faint staining isoenzyme at position 74 which was present immediately after pollen shed and disappeared with as little as 1 hour of storage may indeed prove to be indicative of pollen viability.

Px<sub>3</sub> Locus: The Px<sub>3</sub> peroxidases system was a multiple band complex first observed and studied in mature leaf tissues. This system consisted of a series of anodal bands principally in the positions of isoenzymes 10, 20, 29, 38, and occasionally 51. Two different genetically controlled variants of this pattern were observed among 88 lines tested. One pattern was designated Px<sub>3</sub><sup>1</sup> and had isoenzymes in the positions listed above, while the second pattern, Px<sub>3</sub><sup>2</sup> was composed also of four (or five) bands which were displaced toward the anode to positions 19, 27, 35, 42, and occasionally 51 (Figure 4). The Px<sub>3</sub> peroxidases showed faintly in seedling and young leaves, and reached their maximum clarity in mature leaves which had stopped elongating. The same series of bands was also present in the leaf sheath, faint in young sheaths and more distinct as the sheath completed elongation. Husk material also displayed these isoenzymes.

The two Px<sub>3</sub> patterns, 1 and 2, could be further subdivided into two classes on the basis of intensity of staining of the bands. In one pattern, the slowest moving band of the series stained densely and each faster moving band stained a little less intensely producing a series of bands of decreasing intensity. This pattern was called the "a" type. The second pattern had all of the bands appearing to stain with the same intensity and was called the "b" type. Thus there were four isoenzyme patterns seen for the Px<sub>3</sub> peroxidases, 1a, 1b, 2a, and 2b, and

Fig. 4.  $Px_3$  Patterns in Mature Leaves of Maize.  
At Left is the Total Leaf Blade Peroxidase  
Isoenzyme Complement in a  $Px_3$  Homozygote.



these were repeatable for a given inbred. When gels were scanned with a Densichron, model 3853D, it was found that for the "a" type pattern the bands did actually stain less intensely toward the anode. However, for the "b" type pattern, the second band of the series stained much more intensely than the first (slowest) and then the rest of the bands dropped off in staining intensity in a manner similar to the "a" type, but always staining darker than the "a" pattern. Genetic control of the "a"/"b" type polymorphism was not evident in the progenies studied here.

The basis of the multiplicity, and the different staining patterns has not yet been determined. The fact that these isoenzymes develop with age suggests that there might be a progressive aggregation of peroxidase molecules. The amount of aggregation could account for the difference in staining intensities in the two patterns. The fact that the development of these bands was so closely correlated with the cessation of elongation of tissues leads to the supposition that these peroxidase isoenzymes might be acting in the capacity of indoleacetic acid oxidases.

Eighty-eight inbreds were classified as to their  $Px_3$  pattern in leaf (Table 6) and two were selected, M14 (1a) and L289 (2b), for genetic analysis. All classification and analyses were carried out on the leaf subtending the ear of a mature plant. In the segregating progenies, the gels were scored for only 1 and 2 segregations. The "a" and "b" type patterns did not segregate discretely. Patterns 1 and 2 however, segregated as if under control of alleles at a single locus (Table 7), and this locus was designated  $Px_3$ , with alleles  $Px_3^1$  and  $Px_3^2$ .

Table 6.  $Px_3$  Alleles of Leaf and Root in Several Maize Inbreds

Class	Type	Tissue	Inbred
$Px_3^1$	a	Leaf	245, 303B, 318A, 5446, A509, AA4, AA5, AA6, AA7, AA8, AA9, AA10, AA11, AA13, AA14, AA15, AA18, AA20, AA2sh, AA4sh, AA8sh, AA9sh, AA10sh, B37, B37TRF, CM105, GE54, KYS, M14, Mo1W, N28, ND203, T24, Wf9
$Px_3^1$	b	Leaf	5125, 5446, 5490, AA23, B14, C166, H49, K55, Oh43, Oh51A, T20, T36, T47, W64A
$Px_3^2$	a	Leaf	382-11, 426-1, 650, 2132sh, 2277, 5480, A295, AA1, AA16, AA17, AA19, CM104, M113, M119B, M822A, P51, T55,
$Px_3^2$	b	Leaf	190a, 304A, 442, 647, 661, 2256, 6053, AA2, AA3, AA12, AA17, AA21, AA22, AA3sh, C42, C53, C103, L289, MS1334, NC236, P39, T23, T115
$Px_3^1$	-	Root	451, 5446, 5490, 6053, A257, A545, AA4, AA8, AA9, AA10, AA11, B10, B37, B37TRF, H49, M14, Mo51R, P8, R109, R902, T24, W22
$Px_3^2$	-	Root	2132sh, 2277, 5480, A239, A295, AA3, AA15, AA16, AA17, AA18, AA20, AA21, B8, B190, C123, I153, L289, M119B, MVR6, N6D, N24, R2, R53, R84, R117, R151, R168, R172, T55, Va35, W32, W153R

Table 7. Observed  $F_2$  and Backcross Ratios  
for Crosses Involving Two Alleles at the  $Px_3$  Locus  
in Mature Leaf of Maize

Parentage	Number of Progenies in each Phenotypic Class			Expected Ratio	Total	$\chi^2$	P
	1	2	12				
(M14xL289)x(M14xL289)	19	31	50	1:1:2	100	2.880	.10-.25
(M14xL289)xM14	51	--	41	1:1	92	0.545	.25-.50
(M14xL289)xL289	--	46	51	1:1	97	0.258	.50-.75

The genotype of M14 was thus  $Px_3^1 Px_3^1$  and L289  $Px_3^2 Px_3^2$ .

Isoenzymes which stained medium to faint at these same positions were also seen in older seedling roots. It was determined that these were the same bands as the  $Px_3$ 's of the leaf since there was good correspondence of leaf and root bands in many inbreds. It was not possible to distinguish the "a" and "b" types in root tissues. Roots of  $F_2$  and backcross progenies from two different crosses were analyzed for  $Px_3$ . The first cross involved A295 ( $Px_3^2$ ) and M14 ( $Px_3^1$ ) and the second cross involved H49 ( $Px_3^1$ ) and A295 ( $Px_3^2$ ). As in the analysis of the leaf the  $F_2$  and backcross roots from each cross gave ratios which would be expected if each pattern were conditioned by one allele of a single locus (Table 8). Since the  $Px_3$  bands of root and leaf showed identical positions and inheritance patterns, it was concluded that the same gene was responsible for  $Px_3$  in both tissues.

$Px_4$  Locus: In the course of the analysis of the  $Px_3$  peroxidases in leafy tissues, it was observed that a few inbreds had a distinct band at a position of -18, designated  $Px_4$ . This isoenzyme was quite close to band -20 and sometimes was obscured by it. However, a few inbreds were found to have this band very distinctly staining; these were 303B, 442a, A295, AA12, AA14, B14, B37, C53, KYS, M14, MolW, P39, and T19, while inbreds L289, T24, and T55 very clearly lacked this isoenzyme. Other inbreds (those listed in Table 6), were not classified as to presence or absence of  $Px_4$  because positive identification was not possible due to indistinct staining. This band was not studied in detail in any other tissue except leaves, although there was some indication that it was present also in roots. M14 ( $Px_4$  present) and

Table 8. Observed  $F_2$  and Backcross Ratios  
for Crosses Involving Two Alleles at the  $Px_3$  Locus  
in Seedling Root of Maize

Parentage	Number of Progenies in each Phenotypic Class			Expected Ratio	Total	$\chi^2$	P
	1	2	12				
(M14xA295)x(M14xA295)	12	20	28	1:1:2	60	2.400	.25-.50
(M14xA295)xM14	15	--	23	1:1	38	1.685	.10-.25
(M14xA295)xA295	--	26	34	1:1	60	1.065	.25-.50
(H49xA295)x(H49xA295)	20	22	40	1:1:2	82	0.109	.90-.95
(H49xA295)xH49	27	--	40	1:1	67	2.562	.10-.25
(H49xA295)xA295	--	14	16	1:1	30	0.133	.50-.75



L289 ( $Px_4$  absent) were crossed and the  $F_1$  was observed to lack the  $Px_4$  peroxidase band. The  $F_2$  and backcrosses of the cross were produced and segregations were counted on mature leaves. The ratios are those expected if the  $Px_4$  peroxidase is controlled by a single gene ( $Px_4$ ) with presence-absence alleles, with the absence or null condition being dominant (Table 9). The presence of two plants with the  $Px_4$  isoenzyme in the backcross to the dominant homozygote was ascribed to contamination.

These same ratios would be obtained however, if the presence of  $Px_4$  were governed by a dominant allele of a structural gene which was under the control of a dominant independently segregating regulatory locus. Regulatory loci of other isoenzyme loci have been described in maize for the  $E_1$  esterase (Schwartz 1962) and  $E_4$  esterase (Macdonald 1969). In this situation, there is more than one genotype which will produce the null phenotype. This possibility should be examined for the  $Px_4$  peroxidase.

$Px_5$  Locus: The  $Px_5$  peroxidase isoenzyme stained lightly and moved to a position -24 in mature leaf extracts. Only a single inbred, L289, of those surveyed here was found to lack this isoenzyme in leaf material (Table 10). In the cross M14 ( $Px_5$  present) x L289 ( $Px_5$  absent), the  $F_1$  was found to have the band. The  $F_2$  and backcross progenies gave ratios which indicated that, like  $Px_4$ , the  $Px_5$  peroxidase was due to one locus with presence-absence alleles. However, presence of the  $Px_5$  band was dominant over absence (Table 11). Here also in one backcross there were two individuals with an unexpected phenotype which were assumed to be contaminants. The genotype of M14 and L289 with

Table 9. Observed  $F_2$  and Backcross Ratios  
for Crosses Involving the  $Px_4$  Peroxidase  
in Mature Leaves of Maize

Parentage	Number of Progenies in each Phenotypic Class		Expected Ratio	Total	$\chi^2$	P
	Present	Absent				
(M14xL289)x(M14xL289)	28	72	1:3	100	0.480	.25-.50
(M14xL289)xM14	41	54	1:1	95	1.786	.10-.25
(M14xL289)xL289	2	95	0:1	97	--	--

Table 10.  $Px_5$  Patterns in Mature Leaves of 64 Maize Inbreds

Class	Inbred
$Px_5$ Present	190a, 245, 304A, 318A, 382-11, 426, 442, 647, 650, 661, 2277, 5125, 5446, 5480, 5490, 6053, A295, AA1, AA2, AA3, AA4, AA5, AA6, AA7, AA8, AA9, AA10, AA11, AA12, AA13, AA14, AA15, AA16, AA17, AA18, AA19, AA20, AA21, AA22, AA23, B37, C42, C103, CM105, F6, GE54, H55, KYS, M14, M113, M119B, M822A, N28, Oh51A, P39, P51, T19, T20, T23, T24, T36, T47, T55
$Px_5$ Absent	L289

Table 11. Observed  $F_2$  and Backcross Ratios  
for Crosses Involving the  $Px_5$  Peroxidase  
in Mature Leaves of Maize

Parentage	Number of Progenies in each Phenotypic Class		Expected Ratio	Total	$\chi^2$	P
	Present	Absent				
(M14xL289)x(M14xL289)	73	27	3:1	100	0.214	.50-.75
(M14xL289)xM14	93	2	1:0	95	--	--
(M14xL289)xL289	51	46	1:1	97	0.258	.50-.75

respect to loci  $Px_4$  and  $Px_5$  was thus determined to be  $Px_4^+Px_4^+ Px_5^+Px_5^+$  and  $Px_4^0Px_4^0 Px_5^0Px_5^0$  respectively.

Linkage Studies with  $Px_3$ ,  $Px_4$ , and  $Px_5$ :  $Px_3$ ,  $Px_4$ , and  $Px_5$  were all studied in progenies produced from the cross M14 x L289, and were all analyzed in mature leaf tissues, and hence linkage of these three genes could be tested. Segregations obtained for loci  $Px_3$  and  $Px_4$  in the  $F_2$  and backcross to the homozygous recessive parent provides evidence of linkage, since ratios would be expected to differ depending on linkage. These expected and observed ratios are documented in Table 12. Chi square values for the  $F_2$  indicated that the ratio obtained was within the range of that expected for two genes not linked. The backcross data did not provide such a good fit to the expected ratio. However, classes of progeny were obtained which would not have been seen if the genes were linked and it was concluded that  $Px_3$  and  $Px_4$  were not linked.

When  $Px_3$  and  $Px_5$  segregations were considered (Table 13), the  $F_2$  and critical backcross ratios fit closely those which would have been expected if the two loci were not linked.

It was not possible to obtain evidence on linkage of loci  $Px_4$  and  $Px_5$  from backcross data because of the way in which the cross was set up, with each parent having one of the genes homozygous dominant and one homozygous recessive. Linkage would not be expected to affect segregation ratios in these backcrosses. In the  $F_2$  however, if the two genes were closely linked, the progeny should segregate 1 ( $Px_4^+Px_5^+$ ): 2 ( $Px_4^0Px_5^+$ ) : 1 ( $Px_4^0Px_5^0$ ) and the class  $Px_4^+Px_5^0$  would have occurred only as a result of a crossover. Two non-linked loci should give segregation ratios 9 ( $Px_4^0Px_5^+$ ) : 3 ( $Px_4^+Px_5^+$ ) : 3 ( $Px_4^0Px_5^0$ ) : 1 ( $Px_4^+Px_5^0$ ), and this is

Table 12. Observed and Expected Segregations  
of  $Px_3$  and  $Px_4$  Peroxidases Considered Together

		Number in each Phenotypic Class						Total	$\chi^{2**}$	P
		$Px_3^1 Px_4^0$	$Px_3^1 Px_3^2 Px_4^0$	$Px_3^2 Px_4^0$	$Px_3^1 Px_4^+$	$Px_3^1 Px_3^2 Px_4^+$	$Px_3^2 Px_4^+$			
$F_2$	(M14xL289)x(M14xL289)									
	Expected ratio for 2 non-linked genes	3	6	3	1	2	1			
	Expected ratio for 2 linked genes	--	2	1	1	--	--			
	Observed number in each class	15	27	29	5	21	3	100	6.36	.25-.50
BC	(M14xL289)xM14									
	Expected ratio for 2 non-linked genes	1	1	--	1	1	--			
	Expected ratio for 2 linked genes	--	1	--	1	--	--			
	Observed number in each class	37	13	--	13	27	--	90	18.30	.005

\* $Px_4^0$  = absence of  $Px_4$  isoenzyme.

$Px_4^+$  = presence of  $Px_4$  isoenzyme.

\*\* $\chi^2$  and P are calculated for ratios based on absence of linkage.

Table 13. Observed and Expected Segregations of  
Px<sub>3</sub> and Px<sub>5</sub> Peroxidases Considered Together

	Number in each Phenotypic Class						Total $\chi^2$ **		P
	Px <sub>3</sub> <sup>1</sup> Px <sub>5</sub> <sup>+</sup> *	Px <sub>3</sub> <sup>1</sup> Px <sub>3</sub> <sup>2</sup> Px <sub>5</sub> <sup>+</sup>	Px <sub>3</sub> <sup>2</sup> Px <sub>3</sub> <sup>+</sup>	Px <sub>3</sub> <sup>1</sup> Px <sub>5</sub> <sup>0</sup>	Px <sub>3</sub> <sup>1</sup> Px <sub>3</sub> <sup>2</sup> Px <sub>5</sub> <sup>0</sup>	Px <sub>3</sub> <sup>2</sup> Px <sub>5</sub> <sup>0</sup>			
F <sub>2</sub> (M14xL289)x(M14xL289)									
Expected ratio for 2 non-linked genes	3	6	3	1	2	1			
Expected ratio for 2 linked genes	1	2	--	--	--	1			
Observed number in each class	13	37	24	6	13	7	100	3.35	.50-.75
BC (M14xL289)xL289									
Expected ratio for 2 non-linked genes	--	1	1	--	1	1			
Expected ratio for 2 linked genes	--	1	--	--	--	1			
Observed number in each class	--	23	28	--	26	20	97	1.51	.50-.75

\*Px<sub>5</sub><sup>+</sup> = Presence of Px<sub>5</sub> isoenzyme.

Px<sub>5</sub><sup>0</sup> = Absence of Px<sub>5</sub> isoenzyme.

\*\* $\chi^2$  and P are calculated for ratios based on absence of linkage.

very close to what was observed (Table 14). This strongly indicated that loci  $Px_4$  and  $Px_5$  were not closely linked.

In summary, the data from this set of crosses provided no evidence of linkage of the three loci  $Px_3$ ,  $Px_4$ , and  $Px_5$ .



Table 14. Observed and Expected Segregations of  
Px<sub>4</sub> and Px<sub>5</sub> Peroxidases Considered Together

	Number in each Phenotypic Class				Total	$\chi^2$ **	P
	Px <sub>4</sub> <sup>0</sup> Px <sub>5</sub> <sup>+</sup>	Px <sub>4</sub> <sup>+</sup> Px <sub>5</sub> <sup>+</sup>	Px <sub>4</sub> <sup>0</sup> Px <sub>5</sub> <sup>0</sup>	Px <sub>4</sub> <sup>+</sup> Px <sub>5</sub> <sup>0</sup>			
F <sub>2</sub> (M14xL289)x(M14xL289)							
Expected ratio for 2 non-linked genes	9	3	3	1			
Expected ratio for 2 linked genes	2	1	1	-			
Observed number in each class	53	20	19	8	100	0.802	.75-.90

\*Px<sub>4</sub><sup>0</sup> = absence of Px<sub>4</sub> isoenzyme.

Px<sub>4</sub><sup>+</sup> = presence of Px<sub>4</sub> isoenzyme.

Px<sub>5</sub><sup>0</sup> = absence of Px<sub>5</sub> isoenzyme.

Px<sub>5</sub><sup>+</sup> = presence of Px<sub>5</sub> isoenzyme.

\*\* $\chi^2$  and P are calculated for ratios based on absence of linkage.

## GENERAL DISCUSSION

In the course of this study the multiplicity of peroxidase isoenzymes in Zea mays was demonstrated and the mode of inheritance of five of these systems was determined. As has been reported for several species (Literature Review), the maize isoenzymes displayed both substrate and tissue specificities.

The molecular basis for such heterogeneity can be due to several factors and this subject has recently been reviewed by Markert (1968). The  $Px_1$ ,  $Px_2$ ,  $Px_3$ ,  $Px_4$ , and  $Px_5$  peroxidases are each products of a different locus, and thus probably differ in primary structure. The different isoenzymes of the  $Px_3$  series differed in migration in a regular stepwise fashion from each other, as though there were perhaps progressive masking or revealing of charged groups through aggregation or some other conformational phenomenon. Definite genetic polymorphisms were found for only five of the twenty-one peroxidase bands observed in this study, while two other polymorphisms (6 and 74) appeared to have genetic variants. No doubt others will also be elucidated as more material is investigated.

The possibility of isoenzyme forms being artifacts of either extraction or electrophoretic procedures is definitely ruled out when the isoenzymes are determined to be products of separate genes, as was the case with the five isoenzyme systems here. It appears certain that none of these isoenzymes were artifacts, since the same results on gels were obtained from frozen or fresh samples extracted in several different ways, and since the results obtained here were highly repeatable, even over periods of years. Peroxidase is a notably stable

enzyme (Saunders, Holmes-Siedel, and Stark 1964).

Since multiple molecular forms of enzymes are so common, and found universally in organisms studied, it would seem that they often provide some advantage to the organism. Each isoenzyme may have a specific function and a specific rate of activity under given conditions. As the inheritance of isoenzyme polymorphism becomes known, it is possible to look at each system individually. The tissue specificity alone suggests different functions, and through examination of isoenzymes in light of where they occur and the metabolic and biochemical events occurring within that tissue, it may be possible to infer their role. Mutants affecting growth and normal development, for example, dwarfs, tassel seed, corn grass, etc. will be useful tools for such studies.

Maize pollen presented a highly characteristic unique peroxidase isoenzyme pattern in two respects. Freshly shed pollen was seen to have a band at position 74 which faded out as pollen was stored. The  $Px_2$  peroxidases (67 and 62) stained very intensely and very rapidly. They were specifically pollen isoenzymes, and were not found in any other tissues; furthermore, no null phenotype was observed in this study. An interesting follow up study will be to determine if any of the pollen peroxidases have a role in the pollen tube germination and fertilization processes. Maize pollen loses viability rapidly and if peroxidase activity is truly an indication of viability, isoenzyme 74 in pollen may be the indicator. Clearly peroxidase activity alone cannot be used to measure pollen viability in maize.

The  $Px_3$  peroxidases were observed only in tissues which were undergoing much elongation, leaf blade, sheath, husk and root, and were

not seen in non-elongating tissues such as pollen and endosperm. Moreover, the isoenzyme pattern reached its full complexity as the tissue ceased elongating. This observation suggests that the  $Px_3$  peroxidases may be functioning here as an indoleacetic acid oxidase. Future studies should include the characterization of peroxidase isoenzymes with regard to their indoleacetic acid oxidase activity, either by devising a more satisfactory stain for indoleacetic acid oxidase activity on a gel (Endo 1968), or by eluting the enzyme from the gel and assaying this. Indoleacetic acid treatments in tissue culture experiments have been shown to repress the appearance of certain isoenzymes (Ockerse, Siegel, and Galston 1966). It will be interesting to test the effect of hormone treatments on the  $Px_3$  isoenzymes.  $Px_3$  peroxidases may also be implicated in the lignification process in addition to or instead of the indoleacetic acid oxidation process. Proof of this will require further elucidation of the pattern of lignification in corn tissues.

In a practical sense, isoenzymes may be used as a "fingerprint" method of identifying maize inbreds. Considering only the five  $Px$  loci described here,  $Px_1$  has four alleles, giving four homozygous phenotypes, and  $Px_2$ ,  $Px_3$ ,  $Px_4$ , and  $Px_5$  each have two alleles, providing two homozygous phenotypes. This gives a total of  $4 \times 2 \times 2 \times 2 \times 2$  or 64 different possible peroxidase phenotypes for a homozygous inbred. If one then considers the other eighteen known loci of maize (Table 15); two with 7 alleles ( $E_1$  and  $E_4$ ), three with 3 alleles ( $E_9$ ,  $Cx$ , and  $Phos_4$ ), and thirteen with 2 alleles ( $E_3$ ,  $E_5$ -I,  $E_5$ -II,  $E_6$ ,  $E_7$ ,  $E_8$ ,  $E_{10}$ ,  $Ta_1$ ,  $Adh_1$ ,  $Adh_2$ ,  $LpA$ ,  $LpD$ , and  $Cat$ ), the total number of different possible phenotypes for any one inbred is  $64 \times 7^2 \times 3^3 \times 2^{13}$  or

Table 15. Isoenzyme Loci in Maize (Zea mays L.)

Enzyme	Symbol	Alleles	Tissue	Reference
Alcohol Dehydrogenase	Adh <sub>1</sub>	2	Endosperm and scutellum	Schwartz 1966
	Adh <sub>2</sub>	2	Endosperm and scutellum	Schwartz 1966
Aminotransferase	*Ta <sub>1</sub>	2	Seedling tissue	Macdonald 1969
Catalase	*Cat	2	Endosperm	Beckman, Scandalios, and Brewbaker 1964b
Catechol oxidase	Cx	3	Seedling shoot	Pryor 1969
Esterase	*E <sub>1</sub>	7	Endosperm and seedling	Schwartz 1960b
	*E <sub>3</sub>	2	Seedling tissues	Schwartz 1964
	E <sub>4</sub>	7	Root	Harris 1966; Macdonald 1969
	E <sub>5</sub> -I	2	Root	Macdonald 1969
	E <sub>5</sub> -II	2	Root	Macdonald 1969
	E <sub>6</sub>	2	Root	Macdonald 1969
	E <sub>7</sub>	2	Root	Macdonald 1969
	E <sub>8</sub>	2	Root	Macdonald 1969
	E <sub>9</sub>	3	Root	Macdonald 1969
	E <sub>10</sub>	2	Endosperm	Macdonald 1969

Table 15. (Continued) Isoenzyme Loci in Maize (Zea mays L.)

Enzyme	Symbol	Alleles	Tissue	Reference
Leucine aminopeptidase	LpA	2	Endosperm	Beckman, Scandalios, and Brewbaker 1964a
	LpD	2	Endosperm	Beckman, Scandalios, and Brewbaker 1964a
Peroxidase	Px <sub>1</sub>	4	Root	Hamill 1968
	Px <sub>2</sub>	2	Pollen	Hamill, this thesis
	Px <sub>3</sub>	2	Leaves and root	Hamill, this thesis
	Px <sub>4</sub>	2	Leaves	Hamill, this thesis
	Px <sub>5</sub>	2	Leaves	Hamill, this thesis
Phosphatase	Phos <sub>4</sub>	3	Seedling tissues	Brown and Allard 1969

\*Hybrid bands occur in heterozygotes.

693, 633, 024. The chance of two inbreds having identical "fingerprint" patterns is thus very small. The isoenzyme technique will be of increasing value as a tool for the geneticist, physiologist, biochemist, and breeder.

## SUMMARY

Peroxidase isoenzyme variation in selected tissues of approximately 300 different inbred lines, tropical races and genetic stocks of Zea mays was analyzed using starch gel electrophoretic techniques. Within the control inbred line, AA8, a total of twenty-one bands were observed in all tissues, twelve moving to the anode and nine to the cathode at pH 8.1. Some of these isoenzymes were not stainable with a guaiacol donor; all bands stained similarly with benzidine or o-dianisidine. Many variations were noted among tissues, and within most tissues the isoenzyme pattern changed during growth and development of the tissue.

Genetic polymorphisms in diverse stocks were found for five of the isoenzymes of AA8, making a total of twenty-eight different peroxidase isoenzymes present in all the lines studied. The mechanism of inheritance of these five systems was determined, and five peroxidase loci ( $Px_1$ - $Px_5$ ) were designated. The  $Px_1$  locus, analyzed in roots, comprised four alleles, one of which was a null, while the other three produced isoenzymes of varying electrophoretic mobilities at pH 8.1 on starch gel. Two alleles were identified at the  $Px_2$  locus, each governing the production of a pollen-specific isoenzyme. The  $Px_3$  locus was studied in leaf and root tissues and consisted of two alleles, each allele giving rise to a family of four or five bands which stained most intensely in fully elongated tissues. The  $Px_4$  polymorphism found in leaf, was conditioned by a monogenic system composed of "presence-absence" alleles, with absence dominant.  $Px_5$  was also studied in leaves and was also due to a monogenic "presence-absence" system but with presence dominant. There was no evidence of linkage between  $Px_3$ ,  $Px_4$ , and  $Px_5$ .



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